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Exploring the eDNA dynamics of the host-pathogen pair *Pacifastacus leniusculus* (Decapoda) and *Aphanomyces astaci* (Saprolegniales) under experimental conditions

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Abstract

The oomycete *Aphanomyces astaci* causes crayfish plague, a disease threatening native European crayfish. It is carried and transmitted by American crayfish species, which are the original hosts of *A. astaci*. In recent years, environmental DNA (eDNA) methods have been successfully implemented to monitor the spread of both *A. astaci* and its hosts. However, still little is known about how population density and other environmental factors influence the detectability of this host-pathogen complex. In a mesocosm experiment, we tested the influence of crayfish density, temperature and food availability on the detectability of eDNA for *A. astaci* and its host, signal crayfish *Pacifastacus leniusculus*. We also compared eDNA results with crayfish population density measured by catch per unit effort (CPUE) from two lakes with varying crayfish density and *A. astaci* prevalence. The mesocosm experiment revealed that a limited set of controlled factors can substantially change the detectable amount of eDNA, even though the physical presence of the target organisms remains the same. In cold, clear water, eDNA quantities of both targets increased far more than in a linear fashion with increased crayfish density. However, the presence of food decreased the detectability of crayfish eDNA, presumably through increased microbial-induced eDNA degradation. For *A. astaci*, where eDNA typically represents living spores, food did not affect the detectability. However, high water temperature strongly reduced it. The increased complexity and variability of factors influencing eDNA concentration under natural conditions, compared to a controlled experimental environment, suggests that establishing a reliable relationship between eDNA quantities and crayfish density is difficult to achieve. This was also supported by field data, where we found minimal correspondence between

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eDNA quantity and CPUE data. A comparison between quantitative real-time PCR (qPCR) analysis and droplet-digital PCR (ddPCR) analysis revealed higher detection success of the targets in field samples when using qPCR. Overall, our results support eDNA as an effective tool for presence-absence monitoring, but it seems less suited for biomass quantification and population density estimates. Detection of *A. astaci* and *P. leniusculus* is not influenced uniformly by respective environmental factors. Consequently, we recommend a strategy of monitoring both targets, where the detection of one may point towards the presence of the other.

Keywords
crayfish plague, ddPCR, environmental biomonitoring, environmental DNA, freshwater crayfish, mesocosm experiment, occupancy modelling

Introduction

The oomycete *Aphanomyces astaci* is a fungal-like water mould that causes crayfish plague, a disease lethal to crayfish indigenous to Europe (Söderhäll and Cerenius 1999). It was first introduced into Europe around 1859 (Alderman 1996 and references therein) and is now widespread throughout Europe, mostly through the introduction of American non-indigenous crayfish species (Holdich et al. 2009). These are natural hosts of *A. astaci* that carry and transmit the disease but, unlike their European counterparts, they usually do not succumb to it as they have evolved natural defence mechanisms against the parasite infections (Söderhäll and Cerenius 1999). *Aphanomyces astaci* has contributed to the drastic decline of indigenous crayfish species throughout Europe and the disease can potentially cause the eradication of most if not all indigenous crayfish species populations in Europe (Holdich et al. 2009). Due to the severe impact on these populations, it is considered a listed disease by the World Organisation of Animal Health (OiE) (OIE 2019) and in Norway (Regulation on animal health requirements for aquaculture animals and products thereof and on the prevention and control of certain diseases in aquatic animals, FOR-2008-06-17-819) and features on the IUCN list of 100 of the world's worst invasive alien species (Lowe et al. 2004). In many European countries, legislation is in place and measures have been implemented to combat further spread of *A. astaci* (Regulation (EU) No 1143/2014) (Jussila and Edsman 2020). These measures include monitoring (Strand et al. 2019; Strand et al. 2020) of the spread of *A. astaci* accompanied by local bans on fishing and strict disinfection regulations. Other measures include eradication of *A. astaci* carrying American non-indigenous crayfish species (Sandodden and Johnsen 2010; Peay et al. 2019), as well as the creation of ark-sites (Brickland et al. 2009) where absence of the disease-agent and non-indigenous crayfish species has to be substantiated.

Environmental DNA (eDNA) monitoring is increasingly used for biomonitoring of species, including both macroorganisms and microorganisms (Leese et al. 2016). For macroorganisms, genetic material in the form of shed or abraded cells and cell-fragments or propagules, such as gametes, is captured on a filter, extracted and analysed (Taberlet et al. 2012; Thomsen and Willerslev 2015; Taberlet et al. 2018), while for microorganisms,

such as the zoospores of *A. astaci*, it can be captured on the filter as live cells (Strand et al. 2011). The eDNA dynamics of *A. astaci* have been studied both experimentally (Strand et al. 2012; Svoboda et al. 2013) and under natural conditions (Strand et al. 2014, 2019; Wittwer et al. 2018) and relative quantification of spores in eDNA samples is possible, enabling the detection of outbreak situations (Strand et al. 2019). In Norway, the surveillance of *A. astaci* has been carried out solely through the use of eDNA monitoring in recent years (Vrålstad et al. 2017; Strand et al. 2019). Additionally, presence-absence monitoring of the pathogen and both the susceptible host and the carrier have yielded good results (Agersnap et al. 2017; Strand et al. 2019; Rusch et al. 2020). However, even though some studies show correlation between population density and eDNA quantity for fish species (Takahara et al. 2012; Doi et al. 2015a; Lacoursière-Roussel et al. 2016; Capo et al. 2019, 2021), no clear correlation has yet been established between the detectable amount of eDNA and crayfish population density (Dougherty et al. 2016; Dunn et al. 2017; Johnsen et al. 2020; but see Chucholl et al. 2021 and Sint et al. 2021). The emission of eDNA seems to be influenced by numerous biotic and abiotic factors (Roussel et al. 2015; Stewart 2019), such as ambient water temperature, life-cycle and corresponding behaviour of crayfish (Dunn et al. 2017).

The host-pathogen pair *Pacifastacus leniusculus* and *A. astaci* are a particularly interesting model for studying eDNA dynamics as crayfish leave relatively low traces of eDNA in the water (Johnsen et al. 2020) compared to *A. astaci*, especially during an outbreak situation (Strand et al. 2019). The overarching hypothesis of our study is that eDNA emitted from *P. leniusculus* correlates with population density/number of individuals. However, we also expect that several factors affect both the emission and detectability of eDNA from *P. leniusculus* and its parasite *A. astaci* in the ambient water. The goal of this study was to test the influence of temperature, food availability and crayfish density on the measurable eDNA amount emitted from *P. leniusculus* and its obligate parasite *A. astaci* in a mesocosm experiment. We expected rising temperatures and access to food to cause increased crayfish activity (Flint 1977; Rusch and Füreder 2015) and more faecal matter and, thus, also increased shedding of eDNA from crayfish. For *A. astaci*, we expected that increased crayfish density would naturally lead to increased amounts of *A. astaci* eDNA, while food availability for the crayfish was not suspected to directly influence the pathogen amounts. Strand et al. (2012) showed that latent carrier signal crayfish released more *A. astaci* spores in temperate (18 °C) water than in cold (4 °C) water. We, therefore, expected that a water temperature close to the previously-described sporulation optimum of *A. astaci* near 20 °C (Alderman and Polglase 1986; Alderman et al. 1987; Diéguez-Uribeondo et al. 1995) would lead to the highest *A. astaci* sporulation and eDNA amounts.

To link the experimental data to a real-life situation, we also included a small field survey where water samples were obtained in parallel with catch per unit effort (CPUE) data from two lakes with varying crayfish density and varying infection load with *A. astaci*. The results from the experiments and field survey will hopefully provide more detailed understanding of eDNA dynamics of the host-pathogen pair and provide knowledge that can help in designing better monitoring programmes involving *A. astaci* and freshwater crayfish.

Materials and methods

Crayfish capture and husbandry

In total, 125 *P. leniusculus* specimens (71 female, 54 male, average total length 109.6 mm ±16.8 mm) were obtained by trapping from two Norwegian lakes (Rødenessjøen and Øymarksjøen) within the Halden watercourse in south-eastern Norway. Crayfish in both lakes have a well-documented history of infection with *A. astaci* (Vrålstad et al. 2011; Strand et al. 2014, 2019). The crayfish were marked both by writing numbers on the carapace and by pricking small holes into the tail-fan in a specific pattern as first described by Guan (1997). After measuring length and determining sex, crayfish were kept in a large communal tank at the aquarium facilities of the Norwegian University of Life Sciences (NMBU), Oslo. Shelters and food were provided. We used aerated tap water that was oxygenated with a large aquarium pump. Temperature and oxygen were measured daily.

Capture, transport and husbandry of crayfish were conducted with permits from the Norwegian Food Safety Authority, the County Governor of Østfold and the Norwegian Environment Agency. This, along with euthanasia at the end of the experiment, was conducted in accordance with the Norwegian Animal Welfare Act (LOV-2009-06-19-97) and EU regulations (EU Directive; 2010/63/EU).

Mesocosm experiment

The experiment was designed as full-factorial to analyse the influence of crayfish density, availability of food and temperature on the detectability of eDNA from *P. leniusculus* and *A. astaci* (Fig. 1). For this purpose, four tanks containing 100 l of water were set up with two different densities of crayfish: 2 crayfish (low density) and 20 crayfish (high density) with two different treatments: 1) food/no food. This series of experiments was carried out over six weeks, with three replicates for each temperature (one week = one replicate, Fig. 1). The three first weeks (replicates) were conducted at high (20 °C) temperature representing summer and the three last weeks (replicates) at low (10 °C) temperature representing spring/autumn conditions in Norway. Food was provided in the beginning of the week to one tank of each density. The fed crayfish in the low and high density tanks were given 2 and 20 frozen peas and ½ and 10 frozen shrimps (*Pandalus borealis*), respectively.

For each week, crayfish were randomly picked from the communal tank, assigned to an experimental tank and their number-markings were recorded. All crayfish were kept in their respective tanks for one week. In all tanks, crayfish were provided with sufficient shelters made from PVC tubes. After seven days, triplicate water samples of 1 l (3 × 1 l) were taken from each tank using a peristaltic pump (Masterflex I/P, Cole-Parmer, Vermon Hills, USA), tygon tubing (Masterflex), an in-line filter holder (Millipore, Billerica, Massachusetts, USA) and glass fibre filters (47 mm AP25, Millipore) according to Strand et al. (2019). Before sampling at each tank, tap water was pumped

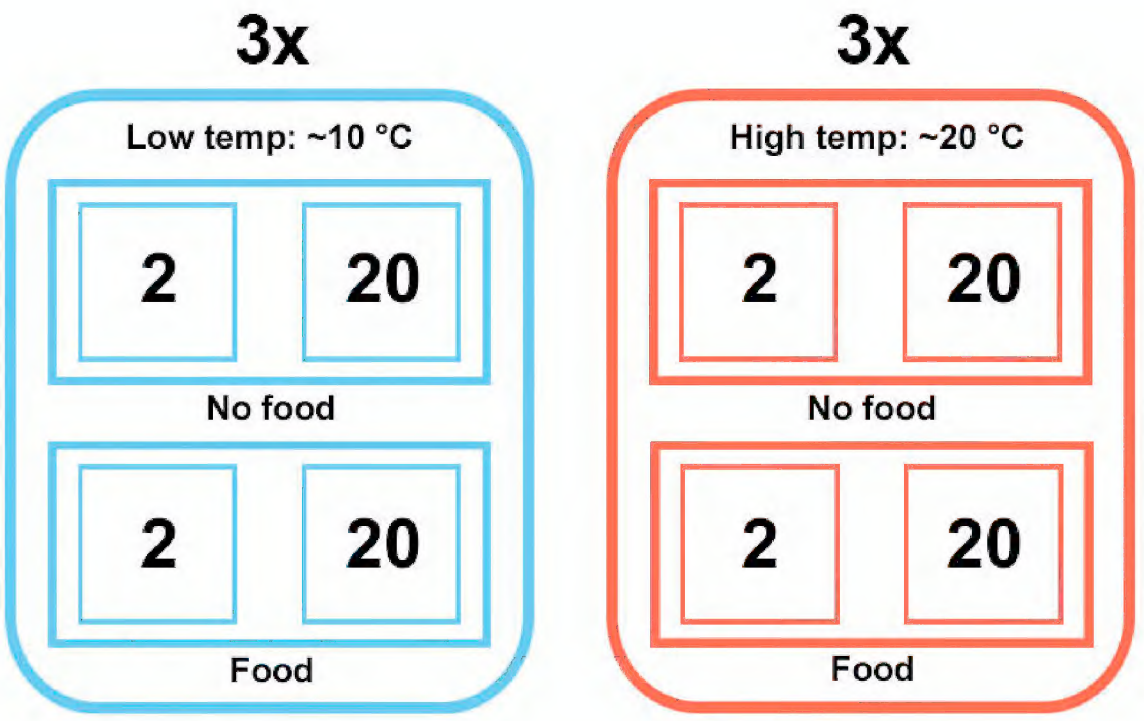

Figure 1. Schematic drawing of the experimental setup with *P. leniusculus* and *A. astaci*. The numbers 2 and 20 represent the number of crayfish present in the respective tanks with 100 l of water. One trial consisted of two tanks with 2 crayfish (low density) and two tanks with 20 crayfish (high density). Crayfish from one tank of each density group were fed, while the crayfish in the parallel tanks got no food. Three replicate trials were run at high (20 °C) water temperature and another three replicate trials at low (10 °C) water temperatures, in total for six weeks.

through the tubes for 5 minutes followed by 1 l of water from the respective tank. At the start of each experiment, negative control eDNA water samples (3 × 1 l) were taken from a clean bucket filled with water from the same source as used in the experiment to check for the presence or absence of eDNA of both *A. astaci* and *P. leniusculus*. Filters were placed in separate sterile 15 ml Falcon tubes and frozen at –20 °C until further analysis. After each sampling date, the tubes were submerged in a 10% chlorine bleach solution which was also pumped through the tubes for a minimum of 10 min. Subsequently, tap water was pumped through the tubes for another 10 minutes, followed by a 10% sodium thiosulphate ($Na_2S_2O_3$) solution to neutralise any residual chlorine. The tubes were then stored at –20 °C until the next sampling date. At the end of each week after sampling, the crayfish were returned to the communal tank, the experimental tanks were drained and the tanks and shelters were scrubbed with detergent and thoroughly cleaned. Using this setup, three replicate trials (always containing a random selection of crayfish individuals from the communal tank) were conducted (Fig. 1).

At the end of the experimental period, the crayfish were euthanised by placing them in ice slush for anaesthesia, followed by piercing of the brain using a scalpel. Tissue samples were taken from the tail-fan of 45 crayfish used in the experiment and analysed with species-specific *A. astaci* quantitative real-time PCR (qPCR) assay for determining the *A. astaci* prevalence and semi-quantitative agent levels, as described in Vrålstad et al. (2009).

Field samples and lakes

Water samples were also obtained from two lakes with well-documented illegally introduced *P. leniusculus* populations (Table 1, Fig. 2). Lake Øymarksjøen (Viken County, eastern Norway) has a surface area of 14.13 km^2 and alien crayfish were first discovered there in 2008 (Vrålstad et al. 2011). Lake Stora Le (Värmland County and Västra Götaland County, western Sweden) has a surface area of 136.1 km^2. *Pacifastacus leniusculus* was first officially reported from three localities in 2002, although, by that time, they had already been in the lake for several years according to local fishermen (Jansson 2017).

In Lake Øymarksjøen, nine sampling sites were selected at which two water samples were collected per site (one in June 2016 and one in August 2016). In order to estimate *P. leniusculus* CPUE, a total of five foldable cylindrical crayfish traps (LiNi) with two entrances and a mesh size of 14 mm (Westman et al. 1978), were set at each site. The traps were baited with raw chicken (Johnsen et al. 2020) and set overnight on 1 September 2016. Thus, the trapping in Lake Øymarksjøen was conducted after eDNA sampling.

Three sampling sites with varying density of crayfish populations were chosen in Lake Stora Le based on previous monitoring (Jansson 2017; Bohman 2020). At each of these three sites, five LiNi traps (Westman et al. 1978; Bergqvist et al. 2016) baited with roach and attached to a line 10 m apart were laid out on 19 September 2016. The traps were lifted the following day and CPUE was estimated. A few hours later, five water samples (5 × 5 l) were taken at each location 10 metres apart, matching the position of the traps, using the filtering protocol described below. We aimed to filter water samples of five litres on site at each sampling location using the same equipment described for the mesocosm experiment, except that we used the Masterflex E/S portable sampler instead. When filters clogged up prior to reaching five litres, the volume of filtered water was recorded (see Table 1). Filters were placed in separate 15 ml Falcon tubes which were stored on ice directly after filtration. Upon arrival at the laboratory, the samples were stored at –20 °C until further analysis.

DNA extraction and eDNA quantification

Before DNA extraction, the filters were frozen at –80 °C and then freeze-dried for 24 h, using a vacuum freeze dryer (Heto drywinner, Thermo Fisher Scientific, Waltham, USA). DNA was extracted from the filters according to a cetyltrimethyl ammonium bromide (CTAB) protocol described in Strand et al. (2019). During extraction, each filter was split into two subsamples (labelled A & B). A laboratory-environmental control and a blank extraction control were included, as described in Strand et al. (2019).

All qPCR analyses were run on a Mx3005P qPCR thermocycler (Agilent, Santa Clara, USA), using the assay for *A. astaci* developed by Vrålstad et al. (2009) and the assay for *P. leniusculus* developed by Agersnap et al. (2017) (Suppl. material 1). We used TaqMan Environmental Mastermix (Thermofisher Scientific, Waltham, US). The qPCR settings for *A. astaci* followed Vrålstad et al. (2009) with modifications to the annealing/extension cycle according to Strand et al. (2014). The qPCR programme for *P. leniusculus* followed the protocol described in Agersnap et al. (2017).

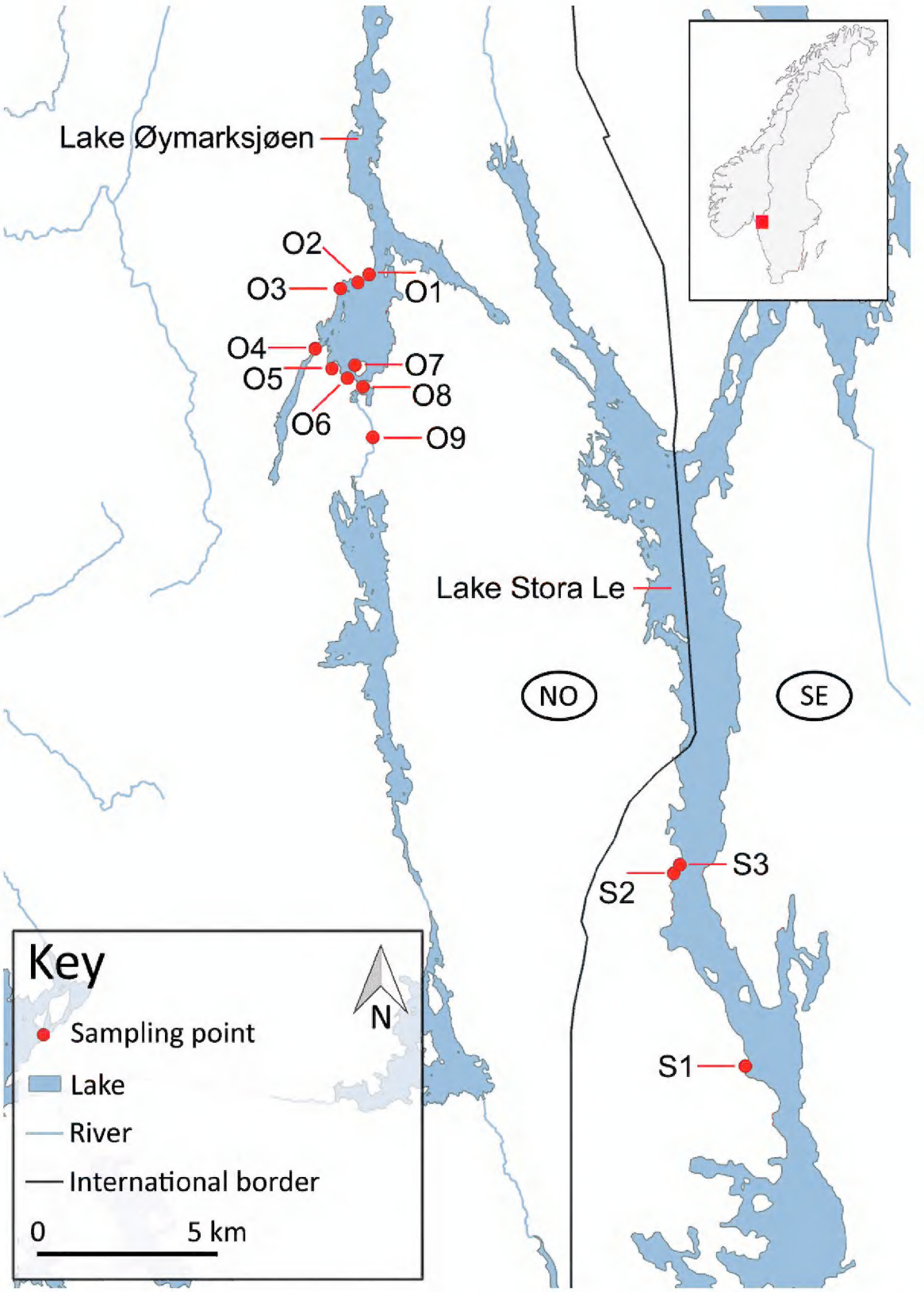

Figure 2. Sampling points in Lake Øymarksjøen in Norway and Lake Stora Le in Sweden. The countries are indicated by their two-letter ISO codes: NO and SE. Sampling points in Øymarksjøen are numbered O1–O9, the sampling points in Lake Stora Le are S1–S3. The respective sampling points are depicted as red dots, the international border is represented by the black line. The location of the map is illustrated by the red area in the inset map in the top right corner.

All DNA isolates were analysed both undiluted and 10-fold diluted to account for potential inhibition, in total four replicates per filter sample. The level of inhibition was determined by calculating the difference in Ct-values between the undiluted and diluted samples (ΔCt) following Kozubíková et al. (2011). In the absence of inhibition, ΔCt theoretically equals 3.32. To account for errors in pipetting, amplification efficiency and other inaccuracies, a variance of 15% was deemed acceptable (ΔCt range

Table 1. List of sampling sites including location, sampling date and amount of water filtered.

Site code	Location	Date sampled	Sample volume (in l)	Coordinates
O1	Øymarksjoen, west of Sandbøl	08.06.2016	5	59.3522N, 11.6608E
		10.08.2016	5	
O2	Øymarksjoen, above Sandbøl	08.06.2016	5	59.3501N, 11.6556E
		10.08.2016	4.5	
O3	Øymarksjoen, south of Sandbøl	08.06.2016	5	59.3483N, 11.6472E
		10.08.2016	5	
O4	Øymarksjoen, Fossbekkbrua	08.06.2016	4.5	59.3331N, 11.6364E
		10.08.2016	4	
O5	Øymarksjoen, hyttefelt	08.06.2016	5	59.3283N, 11.6450E
		10.08.2016	4	
O6	Øymarksjoen, west of Bønesøya	08.06.2016	5	56.3261N, 11.6528E
		10.08.2016	5	
O7	Øymarksjoen, Bønesøya	08.06.2016	5	59.3294N, 11.6561E
O8	Øymarksjoen, Blåsnuppen	08.06.2016	5	59.3242N, 11.6601E
		10.08.2016	2.5	
		10.08.2016	3.5	
O9	Mokallen, outlet to Strømselva	08.06.2016	5	59.3117N, 11.6667E
		10.08.2016	3.5	
S1	Stora Le	20.09.2016	5 (x5)	59.1594N, 11.8625E
S2	Stora Le	20.09.2016	5 (x5)	59.2067N, 11.8231E
S3	Stora Le	20.09.2016	5 (x5)	59.2089N, 11.8261E

2.82 to 3.82). In cases of observed inhibition, the result from the 10-fold diluted subsample was used alone for estimation of eDNA copy number. If the ΔCt range was larger than 3.82, the result from the undiluted subsample was used alone to calculate the eDNA copy number. For subsamples with an accepted range, the mean eDNA copy number per subsample was calculated from the undiluted and 10-fold diluted qPCR result. Reactions with a Ct of 41 or higher were treated as 0 (no detection; Kozubíková et al. 2011; Agersnap et al. 2017).

Genomic DNA from *P. leniusculus* and *A. astaci* with a known DNA copy number concentration was included in each run to create a standard curve for relative quantification of targeted DNA copies in each reaction (Strand et al. 2019) using the manufacturer's software (MXpro, Agilent, Santa Clara, USA). In the following comparisons between qPCR and ddPCR results, the copy numbers per reaction for both DNA extraction subsamples (A and B, listed in Suppl. material 2) were used in the linear regression model (see below).

Droplet digital PCR (ddPCR) was performed on a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, USA). For ddPCR analysis of the samples, we drew upon the qPCR assays developed for *A. astaci* (Vrålstad et al. 2009) and *P. leniusculus* (Rusch et al. 2020) (Suppl. material 1). The positive droplet count and total droplet count per sample are reported by the manufacturer's software (Quantasoft v.1.7.4.0917, Biorad, Hercules, USA). Calculation of eDNA copy numbers per reaction volume is performed by the same software and is estimated using the ratio between

positive and negative droplets within a sample, using Poisson-statistics. We defined a positive detection as ≥ 3 positive droplets in assays with > 8000 total droplets (Dobnik et al. 2015). While the ddPCR eDNA copy numbers for samples with < 3 positive droplets were included in the linear regression comparing ddPCR and qPCR, reactions with < 3 positive droplets were scored as negative in the following statistical analysis and reactions with < 8000 total droplets were scored as missing values (Suppl. material 2). In oversaturated samples, i.e. where the DNA content exceeded the dynamic range of ddPCR quantification with all droplets being positive, we set the maximum amount of DNA copies per reaction to 200,000 for the calculation of copies per litre.

Statistical analysis

We used linear regression on log(x + 1)-transformed variables to investigate the overall consistency between ddPCR- and qPCR-based copy numbers (per reaction) and assessed "goodness of fit" from the Pearson correlation coefficient (r) between the two. We used generalised linear models (GLMs) to estimate effect sizes of the treatments in the laboratory experiments. Since the positive droplet count in a ddPCR assay conforms better to statistical distributions of the exponential families than the non-integer copy number estimates derived from this statistic, we decided to model the logarithm of positive droplets using the logarithm of total droplets as offset (i.e. including a "+ offset(log(tot. drp))" term in the model formula). Using this model construct, we essentially modelled the fraction of droplets that are positive with maintaining a dependent variable that is an integer count. Since this type of data often exhibits more zero counts than expected from a Poisson distribution (so-called over-dispersion), we fitted models of both the Poisson and negative binomial families and compared their performances by Akaike's Information Criterion (AIC). To investigate possible interactive effects between treatments, we fitted models with and without interactions and compared these also by AIC. To account for the pseudo-replication introduced by taking three samples from each tank at the end of each experimental run, we used Tank ID nested within experimental run as a random intercept effect (i.e. including a "+ (1 | Run / Tank)" term in the model formula). We also chose to sum the droplet counts from the A and B filter halves instead of having an additional hierarchical level in the models. We fitted the resulting generalised linear mixed models (GLMMs) with the glmmTMB package (Brooks et al. 2017) (See Suppl. material 3). The glmmTMB is likelihood-based with the same syntax as the older lme4 package, but is known to be faster and more computationally stable due to its use of the Template Model Builder (TMB) automatic differentiation engine (Brooks et al. 2017). All statistical analyses were carried out using R version 4.0.3 (R Core Team 2020).

For the field data, we used 3-level hierarchical occupancy models to represent the variation between sites, between replicated filter samples from the same site and between assays on separate halves of the same filter. In this analysis, we focused on presence of *P. leniusculus* and *A. astaci* eDNA. Here also, a positive detection was defined as ≥ 3 positive droplets in a reaction with > 8000 total droplets (reactions with < 8000 total droplets were flagged as missing values). We fitted the resulting 3-level binomial

models with a Bayesian approach using the msocc package for R (Stratton et al. 2020). We used msocc's default non-informative priors, but increased the number of Markov Chain Monte Carlo samples to 11000, with the first 1000 discarded as warm-up and the remainder thinned by 10 (See Suppl. material 4).

While all DNA, PCR and environmental laboratory controls remained negative in the ddPCR analysis, we experienced low positive signals in some of the inlet water controls in weeks 5 and 6. To test if these weak positive detections influenced the results, we used the same GLMM analysis as described above. All samples collected in the same week as the positive inlet controls that were equal to or lower than the positive control for that week were set to zero. Thus, we used the droplet count of the positive inlet control as the threshold for scoring samples positive. The statistical GLMM tests for the effect of the contamination showed no difference in the significant factors when adjusting for the positive inlets controls (see Suppl. material 5). We, therefore, included all samples from weeks 5 and 6 when analysing the results.

Results

Host & pathogen eDNA in the mesocosm experiment

From the 45 analysed crayfish, representing 36% of the total amount of crayfish used in the experimental population, the prevalence of *A. astaci* was 78% and the agent level varied from A_0 to A_6. According to this classification, agent levels A_0 and A_1 are considered negative, while agent levels A_2 to A_6 indicate presence of the pathogen with exponentially increasing amounts of detectable pathogen DNA (Vrålstad et al. 2009). Tail-fan samples, taken and analysed after the experiment had been concluded, confirmed that all but two tanks and replicates certainly included crayfish individuals with a positive *A. astaci* carrier status (See Suppl. material 6).

Suppl. material 2 summarises ddPCR and qPCR data from the mesocosm experiments, including eDNA copy numbers obtained by both methods. For qPCR analyses of *A. astaci* from the mesocosm experiment samples, we observed inhibition in only two samples ($\Delta Ct < 2.82$), both from tanks containing 2 crayfish with food added, one at low and one at high temperature. For *P. leniusculus* qPCR results, inhibition was observed in seven samples, both with and without food added and at both temperatures (Suppl. material 2). A comparison of the qPCR and ddPCR results from the mesocosm trials yielded a significant positive correlation between the methods for both *A. astaci* (Fig. 3A) and *P. leniusculus* (Fig. 3B). In the following, we use the ddPCR data in the further presentation of results. Corresponding results for qPCR are presented and discussed in Laurendz (2017).

Of the 72 water samples taken during the aquarium experiment and analysed with ddPCR, 46 were positive for *A. astaci* and 60 were positive for *P. leniusculus*. A total of 21 DNA extraction subsamples were excluded from the analysis due to the total droplet count in the reaction being below 8000. The number of positive droplets per ddPCR reaction ranged from 3 to > 19433 (See Suppl. material 2). In the text below, the number of positive droplets represents a proxy for eDNA quantity.

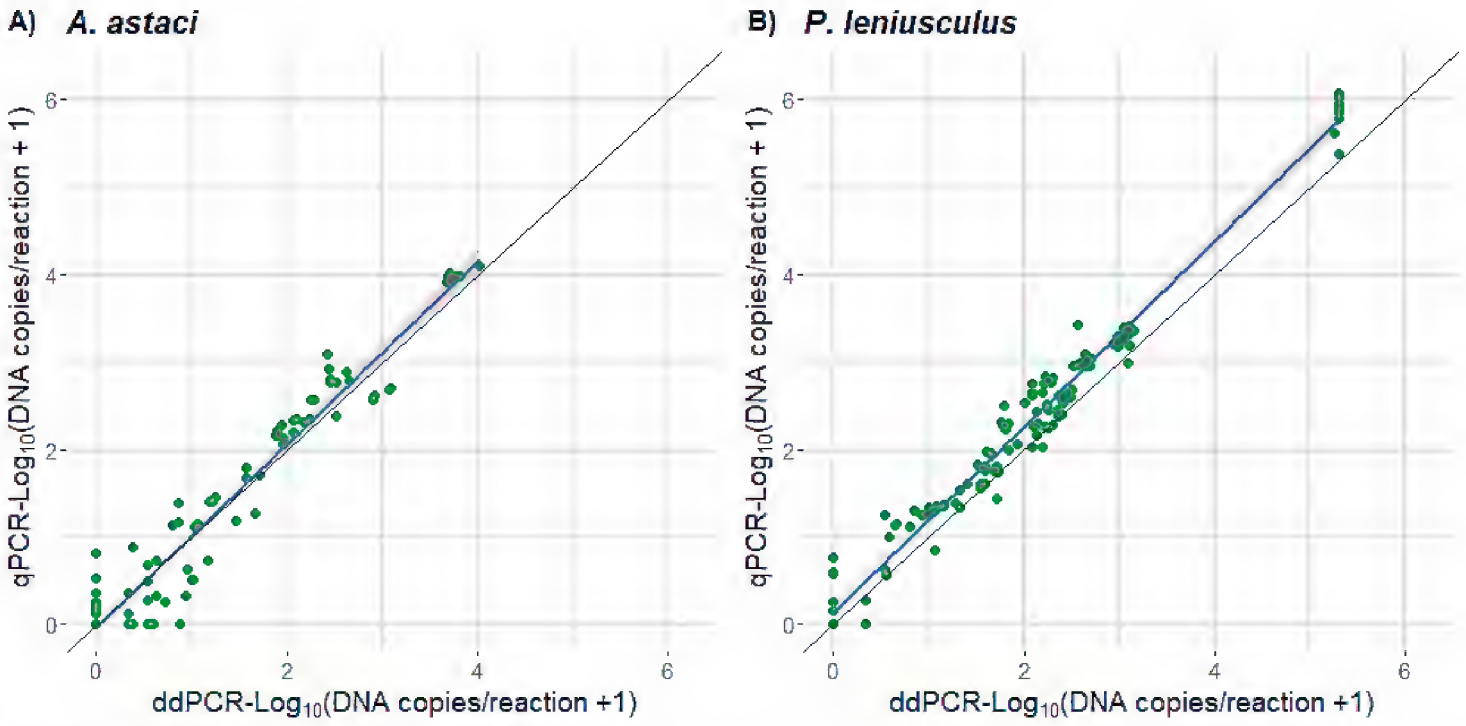

Figure 3. Scatterplot of the estimated DNA copies per reaction of both qPCR and ddPCR analysis for *A. astaci* (**A**) and *P. leniusculus* (**B**) from the mesocosm trial. A significant positive correlation between the methods was observed. A) *A. astaci*: Pearson's r = 0.98, $p < 2.2\times10^{-6}$. B) *P. leniusculus*: Pearsons's r = 0.99, $p < 2.2\times10^{-16}$. Black line represents 1:1 correspondence between ddPCR and qPCR.

For *A. astaci*, the median eDNA copy number per litre was much lower at 20 °C than at 10 °C, irrespective of any other factor/influence (Table 2, Fig. 4). The highest median copy numbers per litre were observed in the 10 °C tanks at high crayfish density, both when food was provided (median eDNA copies per litre = 44556) and when food was missing (median eDNA copies per litre = 28622).

These observations were reflected by the statistical modelling. For *A. astaci*, the two-way interaction model had the lowest AIC value. High crayfish density had a significant positive effect on eDNA quantity (positive droplets), whereas high temperature had a significant negative effect on eDNA quantity of *A. astaci*. The combination of high temperature and high density also had a significant negative effect on the amount of detectable *A. astaci* eDNA (See Suppl. material 3, Fig. 5A).

For *P. leniusculus*, the highest median number of eDNA copies per litre ($> 8.4\times10^{6}$) was observed in the treatment group with high crayfish density and no food at 10 °C. However, the treatment group with high crayfish density and no food at 20 °C had a median of 17467 eDNA copies per litre, lower in fact than the treatment group with low crayfish density and no food at 20 °C (median eDNA copies per litre = 20667) (Table 2, Fig. 4).

The results of the model matched the results of the detected eDNA copy numbers per litre of *P. leniusculus*. Here, the three-way interaction model had the lowest AIC value. High density and the combination of no food at low temperature and high density were determined to have a significant positive effect on the amount of eDNA quantity (positive droplets) by the GLMM model. The two combinations of high temperature with food and high temperature with high density had a significant negative effect (Fig. 5B, Suppl. material 3).

Figure 4. Boxplot of detectable eDNA copies per litre for *A. astaci* (**A**) and *P. leniusculus* (**B**), as detected by ddPCR. For temperature, the blue box indicates the interquartile range at 10 °C, while the red box indicates the interquartile range at 20 °C. Density is indicated by 2 (crayfish per tank) and 20 (crayfish per tank) and the median is represented by the thick black horizontal bar within the boxes. **A** for *A. astaci*, the median copy number/l was generally very low at 20 °C, while high median copy numbers/l were observed at 10 °C and high crayfish density. Food had no apparent effect **B** for *P. leniusculus*, the highest median eDNA copy number/l was observed at high crayfish density at 10 °C, with no food. The median copy number/l was generally substantially lower at 20 °C and, in particular, in the tanks where crayfish were fed. Food had a negative effect on eDNA copy numbers both at 10 °C and 20 °C.

Table 2. Summary of the median eDNA copies per litre with coefficient of variation in brackets of *P. leniusculus eDNA* and *A. astaci* eDNA for the combinations of test conditions: density, food availability and temperature. Fold change indicates the relative increase (x : 1) or decrease (1 : x) in eDNA copy numbers per litre of water from low density (2 crayfish) to high density (20 crayfish).

Temp	Target	Food			No food		
		2 crayfish	20 crayfish	Fold change	2 crayfish	20 crayfish	Fold change
10 °C	*P. len*	5378 (92%)	2533 (21%)	1 : 2.1	8089 (78%)	8488889 (93%)	1049 : 1
20 °C	*P. len*	844 (139%)	1689 (120%)	2 : 1	20667 (94%)	17467 (75%)	1 : 1.2
10 °C	*A. ast*	262 (132%)	44556 (107%)	170 : 1	622 (115%)	28622 (108%)	46 : 1
20 °C	*A. ast*	27 (103%)	0 (210%)	NA	0 (147%)	53 (170%)	NA

The effect of crayfish density on the amount of detected eDNA copies per litre, both for *P. leniusculus* and *A. astaci* eDNA, varied considerably. At 10 °C, we observed a 170-fold increase of the median eDNA quantity (represented by DNA copies per litre) of *A. astaci* from tanks with 2 crayfish to tanks with 20 crayfish provided with food. In the absence of food, a 46-fold increase was observed. At 20 °C, almost no *A. astaci* eDNA was detected in any of the tanks, only trace levels close to or below LOD (3 positive droplets) were observed (Table 2, Fig. 5, Suppl. material 2).

For *P. leniusculus* at 20 °C, we found only a two-fold increase of the median eDNA quantity between the tanks with 2 and 20 crayfish provided with food and even a minor (1.2-fold) decrease when food was missing. At 10 °C, the median eDNA quantity was 2.1 fold lower in the tanks with 20 crayfish compared to 2 crayfish, when food was provided. However, in the absence of food, the median eDNA quantity was as much as 1049-fold higher in the tanks with 20 crayfish compared to 2 crayfish (Table 2, Fig. 5).

Host and pathogen eDNA in natural environments

Of the 15 samples analysed from Lake Stora Le, 10 (66.7%) were positive for *A. astaci* eDNA and 7 (46.7%) were positive for *P. leniusculus* eDNA using ddPCR. Of the 18 samples analysed from Øymarksjøen, 11 (61.1%) were positive for *A. astaci* eDNA, while none was positive for *P. leniusculus* eDNA with ddPCR. For qPCR, 13 (72.2%) samples were positive for *A. astaci* and 12 (66.7%) were positive for *P. leniusculus* eDNA (Table 3).

While there was relatively good correlation between the qPCR and ddPCR results from Lake Øymarksjøen for *A. astaci* (Fig. 6A), the correlation between qPCR and ddPCR results for *P. leniusculus* was weak (Fig. 6B). Here, eight samples that were positive for *P. leniusculus* eDNA using qPCR were recorded as negative with ddPCR.

Using the msocc package, we calculated the statistical probability of detecting *A. astaci* and *P. leniusculus* at crayfish densities ranging from 0 to 20 CPUE, based on the detection rates from field samples (Fig. 7). The probability of presence at site (ψ) is stated for each location and organism respectively in Table 3. The probability of occurrence in the sample (θ), conditional upon presence at site, was 0.69 for *P. leniusculus* and 0.72 for *A. astaci*, respectively. The probability of detection in the filter replicate, conditional upon occurrence in the sample, was 0.86 for *P. leniusculus* and 0.7 for *A. astaci*, respectively.

The probability of detecting eDNA of *A. astaci* using the sampling method described above reached 100% at a crayfish density of 2 CPUE in both Stora Le and Øymarksjøen. For *P. leniusculus*, we calculated a 100% eDNA detection probability above a crayfish density of 5 CPUE in Stora Le. The lack of positive detections in Lake Øymarksjøen using ddPCR provided us with insufficient data points to calculate the detection probability for Lake Øymarksjøen accurately. In the subsequent analysis using qPCR data, we calculated a 100% eDNA detection probability above 3 CPUE in Øymarksjøen. The eDNA concentration in the samples obtained from the field was consistently lower than in the aquarium samples, even in locations with high CPUE.

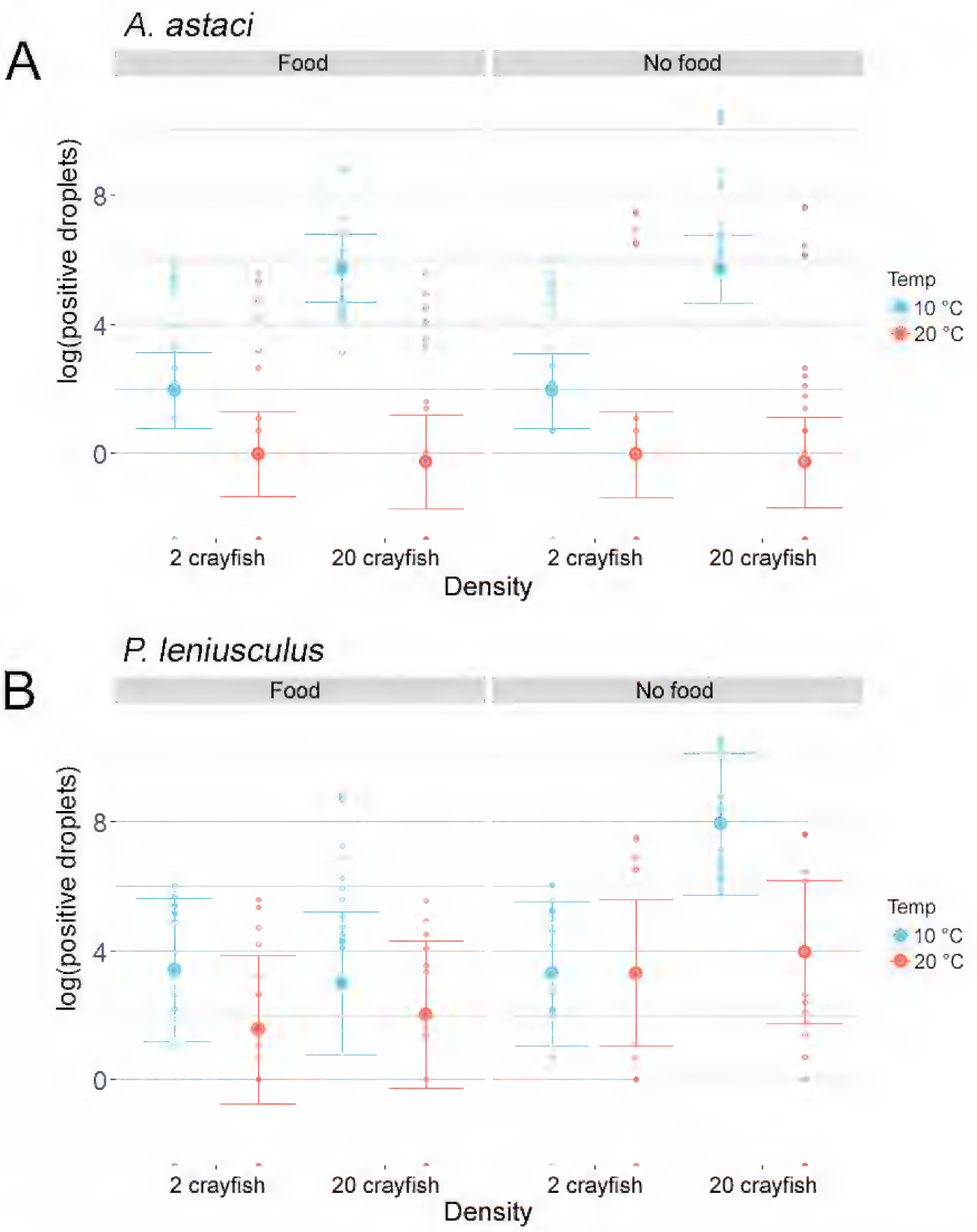

Figure 5. Generalised mixed effect model analysis of the influence of temperature, density and food availability on the amount of detectable eDNA of *A. astaci* (**A**) and *P. leniusculus* (**B**) in the mesocosm experiment. The amount of detectable eDNA is represented as positive droplets per sample (log scale). **A** for *A. astaci*, the eDNA quantity (positive droplets) was significantly higher in tanks with high crayfish density (20 crayfish) at 10 °C, while high temperature (20 °C) had a significant negative effect on the eDNA quantity for all combinations **B** for *P. leniusculus*, the eDNA quantity (positive droplets) was significantly higher for the combination "no food" for 20 crayfish at 10 °C, while the combination 20 crayfish provided with food at 20 °C had a significant negative effect on the eDNA quantity.

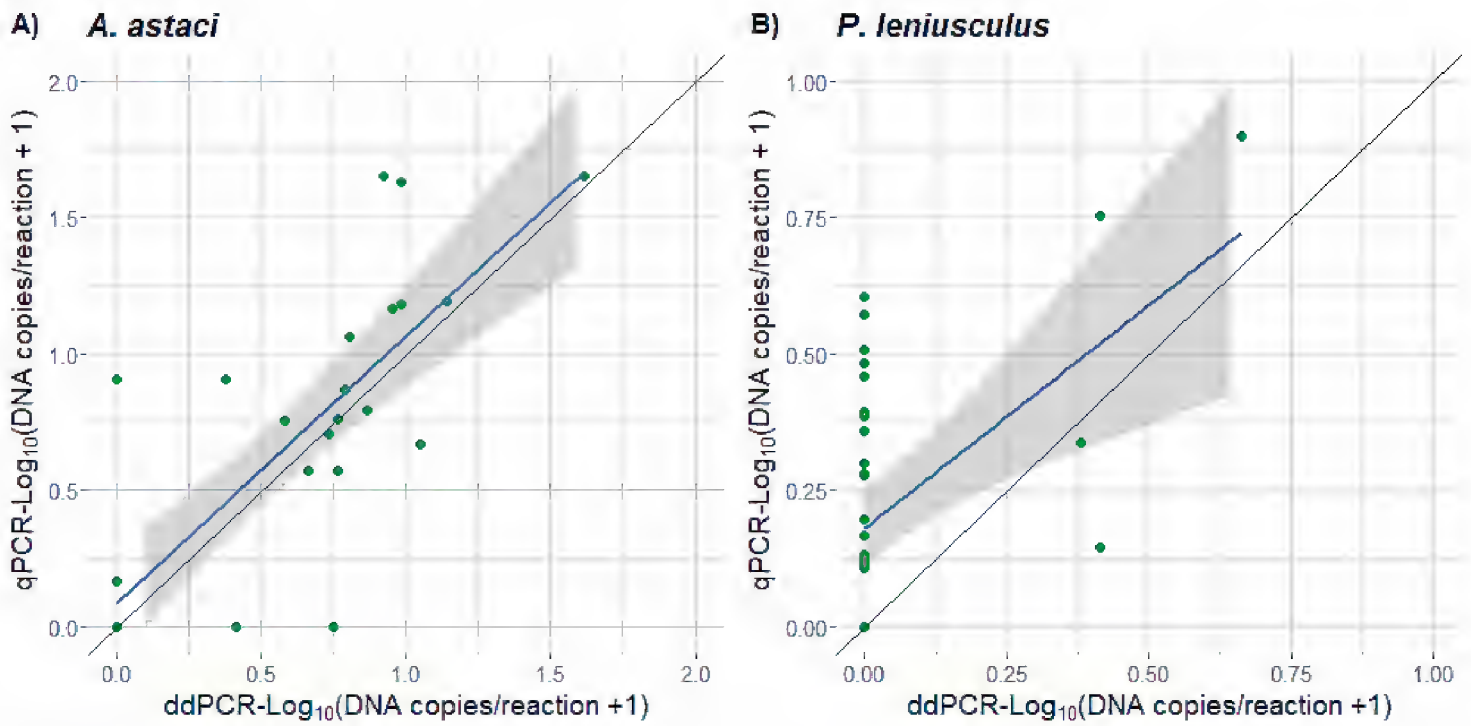

Figure 6. Scatterplot of the estimated DNA copies per reaction of both qPCR and ddPCR analysis for *A. astaci* (**A**) and *P. leniusculus* (**B**) from Lake Øymarksjøen. **A** for *A. astaci*, the correlation between qPCR and ddPCR results is relatively good (Pearson's r = 0.81, p = 2.4×10^{-10}) **B** for *P. leniusculus*, the correlation between qPCR and ddPCR results is poor (Pearson's r = 0.53, p = 0.0011). Black line represents 1:1 correspondence between ddPCR and qPCR.

Table 3. Summary of results from field samples at Lake Stora Le and Lake Øymarksjøen for eDNA detection of *A. astaci* and *P. leniusculus*. The dates of sampling are provided together with the location and sample replicate in Table 1. The volume of water is stated in litres (l) and the catch per unit effort (CPUE) for each respective site is presented. Observed detection-frequency (ω) for both *A. astaci* (*A. ast*) and *P. leniusculus* (*P. len*) is stated. A sample was scored positive for detection if one or both of the two filter subsamples yielded positive amplification of target DNA. The detection probability per site (ψ) as calculated using msocc occupancy modelling is also stated for both organisms.

Lake	Location	# samples	Volume (l)	CPUE	ω *ddPCR / qPCR*		detection probability (ψ)	
					A. ast	*P. len*	*A. ast*	*P. len*
Stora Le								
	S1	5	25	20	0.8 / NA	0.8 / NA	1	1
	S2	5	25	3.6	1 / NA	0.6 / NA	0.99	0.96
	S3	5	25	0.6	0.2 / NA	0 / NA	0.94	0.29
Øymarksjøen								
	O1	2	10	4	0.5 / 0	0 / 0.5	0.99	0.00
	O2	2	9.5	9.6	1 / 1	0 / 0	1	0.00
	O3	2	10	13.2	0.5 / 0.5	0 / 0.5	1	0.00
	O4	2	8.5	17.6	0 / 0.5	0 / 0.5	1	0.00
	O5	2	9	25.4	1 / 1	0 / 1	1	0.00
	O6	2	10	12.2	1 / 1	0 / 1	1	0.00
	O7	1	5	25.8	0 / 0	0 / 0	1	0.00
	O8	3	11	13.2	1 / 1	0 / 1	1	0.00
	O9	2	8.5	6	0 / 1	0 / 1	0.99	0.00

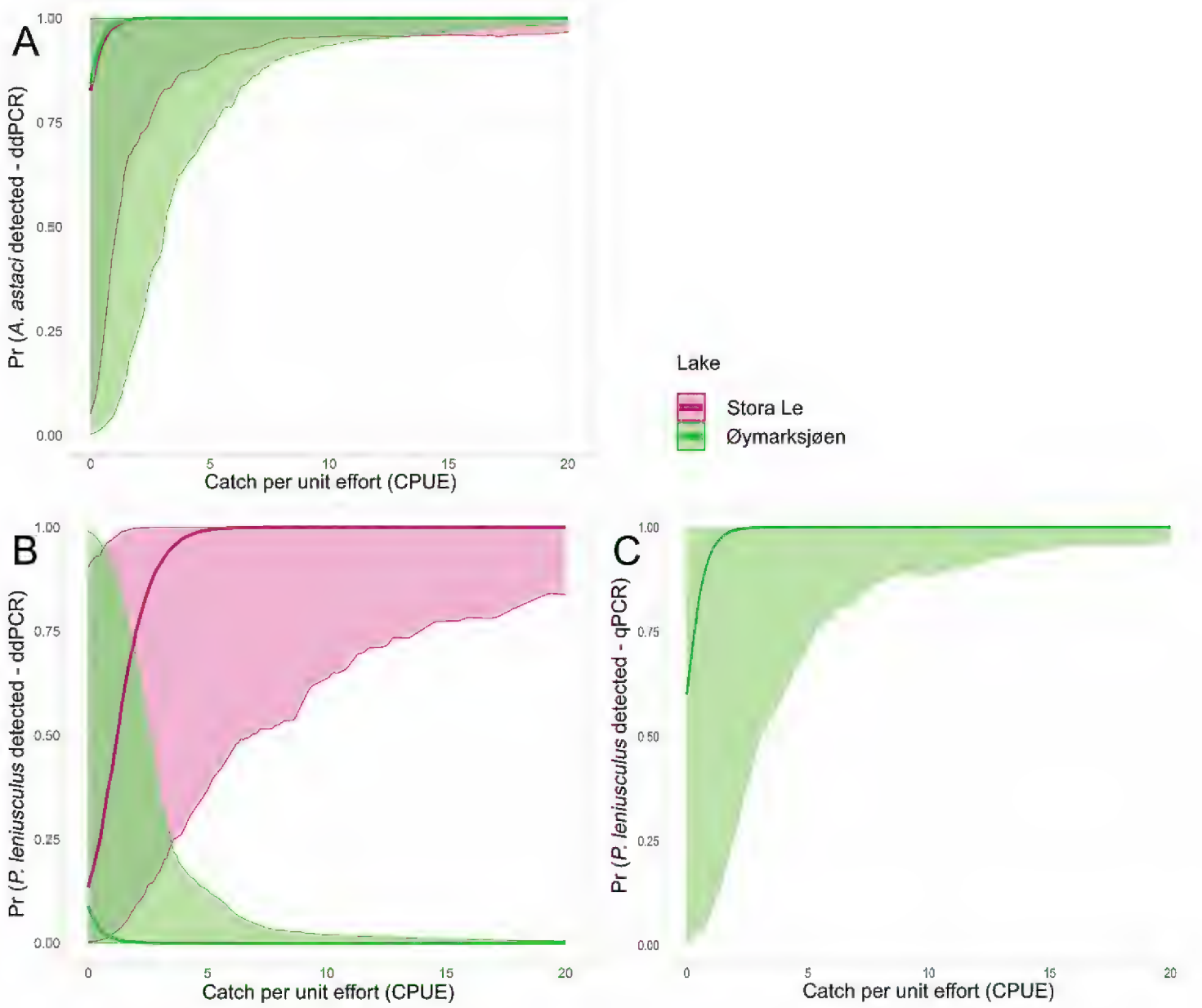

Figure 7. Modelling of probability of detection for *A. astaci* (**A**) and *P. leniusculus* (**B**) with respect to catch per unit effort (CPUE) in lakes Øymarksjøen (green line) and Stora Le (purple line) using msocc, based on ddPCR results. The thick lines represent the median detection probability, while the thin lines represent the upper and lower quantile. The figures are based on 11000 iterations, the first 1000 as warm-up and the rest thinned by 10. Figure 7C depicts the probability of *P. leniusculus* detection in relation to CPUE in Lake Øymarksjøen, based on qPCR results.

Discussion

The mesocosm experiment conducted in our study demonstrates that environmental factors might drastically change the detectable amount of eDNA from *A. astaci* and *P. leniusculus*. In the cold and clear water in the experimental tanks, i.e. in the absence of food supplies, eDNA quantities of *P. leniusculus* and *A. astaci* increased far more than in a linear fashion with crayfish density. However, food availability seemed to contribute to a faster degradation of *P. leniusculus* eDNA. *A. astaci*, on the other hand, was unaffected by the presence of food in the cold water, while a water temperature of 20 °C had a surprisingly huge negative impact on *A. astaci* detectability from eDNA, regardless of food availability.

We found little support for our hypothesis that eDNA emitted from *P. leniusculus* scales directly with the number of individuals. Instead, we observed that small changes to the experimental environment led to large changes – both positive and negative – in

the quantity of detectable eDNA. This indicates that the complexity and variability of influencing factors under field conditions obstructs predictable correlations between eDNA quantities and crayfish density. This is supported by our field data with no clear correlation between eDNA detectability and crayfish population density (as estimated by CPUE). A study on another crustacean, the green crab (*Carcinus maenas*), recently concluded that eDNA cannot be used to rigorously predict the biomass of the target species under controlled conditions (Danziger et al. 2022). The conclusion that eDNA is seemingly not a well-suited tool for the quantification of biomass and population density of *P. leniusculus* is also in concordance with the recently-published study by Johnsen et al. (2020). They demonstrated that high crayfish density was associated with a high detection probability, but not with increased amounts of eDNA.

For surveillance purposes, our study supports a strategy of detecting both the host and the pathogen. As the eDNA detectability of this alien host-pathogen couple seems to be affected differently, eDNA surveillance of both targets will increase the total detection probability, since detection of one may also suggest the presence of the other. This will, of course, only apply in habitats or regions where *A. astaci* is prevalent in alien crayfish hosts and not for American crayfish populations with very low or even missing pathogen prevalence (Schrimpf et al. 2013; Tilmans et al. 2014; Mojžišová et al. 2022).

Under field conditions, eDNA itself and the detectability of eDNA is subjected to a multitude of factors, such as UV radiation, dilution, inhibition through humic acids, retention in substrate and transport that expedite its degradation or disappearance from the system (Jerde et al. 2016; Shogren et al. 2017; Stewart 2019; Wang et al. 2021). In a mesocosm experiment, many of these environmental factors that contribute to fast degradation, masking or disappearance of eDNA are reduced or eliminated. At 10 °C and in the absence of food, we observed an over 1000-fold increase in *P. leniusculus* eDNA and 50-fold increase in *A. astaci* eDNA from a 10-fold increase of crayfish density. While planning the experiment, we expected the availability of food to increase the eDNA concentrations through an increased activity level (Danziger et al. 2022) and faeces production (Ghosal et al. 2018). However, when crayfish were fed, we detected less *P. leniusculus* eDNA in tanks with high crayfish density than in low-density tanks. The water in the high-density tanks with fed crayfish became murkier than in the other tanks and this most likely triggered a much higher microbial activity which can lead to faster degradation of eDNA (Barnes et al. 2014; Barnes and Turner 2016; Salter 2018; Saito and Doi 2021). When measuring eDNA content after a week in this water, a higher degradation of eDNA from *P. leniusculus* might be expected. In contrast to the live *A. astaci* zoospores and even encysted spores (Söderhäll and Cerenius 1999) where the DNA is protected in living cells, the eDNA sources from *P. leniusculus* are more vulnerable to rapid degradation.

Even though sporulation of *A. astaci* has been described as most efficient below 20 °C (Alderman and Polglase 1986; Alderman et al. 1987; Diéguez-Uribeondo et al. 1995) and also observed to decrease above 18 °C (Strand et al. 2012), the drastic reduction of detectable eDNA of *A. astaci* at 20 °C compared to 10 °C was surprising. Strand et al. (2012) observed a negative correlation between temperatures rising from 17 °C to 23 °C and the number of spores produced from infected *P. leniusculus*. A temperature of 10 °C might, therefore, be more conducive to sporulation than temperatures around

20 °C, which seem to be beyond the temperature optimum of the *A. astaci* strain that infected our experimental crayfish. However, this does not fully explain the apparent failure of *A. astaci* spore production in our experiment at 20 °C. Factors both regarding different temperature optimum of different *A. astaci* strains, as well as host differences in the immunity performance at different temperatures, could also have played a role.

The huge increase (> 1000 fold) in eDNA concentrations in the high-density tanks with non-fed crayfish at 10 °C might be explained by injuries from aggressive interactions (Sint et al. 2021) combined with the relatively clean water with assumingly low microbiological activity. In a similar tank experiment, Dunn et al. (2017) successfully detected eDNA of *P. leniusculus*, but only established a significant relationship between eDNA concentration and crayfish biomass when female crayfish were ovigerous. In their study, samples were taken after 11 days. Contrary to our findings and those of Dunn et al. (2017), Harper et al. (2018) observed an increase in eDNA concentration when comparing tanks with one and three *P. leniusculus.* Additionally, Sint et al. (2021) report a clear correlation between eDNA signal strengths and crayfish densities. However, Harper et al. (2018) observed a decrease of eDNA over time, whereas Sint et al. (2021) observed a linear increase during the first three days. While Harper et al. (2018) sampled one, three and seven days after adding crayfish to the tanks and Sint et al. (2021) took multiple samples up to 59 hours after the crayfish had been added to the tanks, the sampling in our experiment and that of Dunn et al. (2017) was conducted after seven and eleven days, respectively. This could have led to a state of saturation or equilibrium where eDNA is emitted from crayfish at a similar rate to its degradation by microbial activity (Barnes et al. 2014; Salter 2018; Saito and Doi 2021), thus obscuring any differences between the tested factors. The short persistence of crayfish eDNA is reflected in the study by Harper et al. (2018). Here, seven days after removal of crayfish, eDNA was detected only in the tanks that had contained three crayfish. Therefore, daily sampling might have revealed more interaction between crayfish density and eDNA concentrations in our study.

When using ddPCR, we observed a relatively good detectability of eDNA from both targets in the field samples in Lake Stora Le and also good detectability of *A. astaci* in Lake Øymarksjøen. Surprisingly, we did not detect *P. leniusculus* in any of the samples from Lake Øymarksjøen with ddPCR, but in 66.7% of the samples when using qPCR. It is unlikely that this was caused by insufficient assay specificity as we obtained satisfactory results from the mesocosm experiment using the same assay on *P. leniusculus* originating from the interconnected lakes Øymarksjøen and Rødenessjøen. However, these results are similar to those in the study by Johnsen et al. (2020) where reduced detection frequency was observed for noble crayfish (*Astacus astacus*) eDNA when using ddPCR compared to qPCR. Inhibition has been reported in another study that screened samples for *P. leniusculus* using ddPCR (Porco et al. 2022). This is in stark contrast to other studies focusing on other organisms than crayfish, that report on higher sensitivity when analysing eDNA samples with ddPCR compared to qPCR (Doi et al. 2015b; Mauvisseau et al. 2019; Wood et al. 2019; Brys et al. 2021). With the exception of the samples from Lake Øymarksjøen analysed for *P. leniusculus*, we found a good correlation between qPCR and ddPCR results, both in field samples and in the mesocosm experiment, but the correlation was unquestionably much bet-

ter in the mesocosm experiment, pointing towards environmental factors in lakes that might impact negatively on the ddPCR results.

The overall detection rate for both organisms was higher in Lake Stora Le than in Lake Øymarksjøen. A speculative explanation is that this may result from trapping (for logistical reasons) prior to sampling in Lake Stora Le. Ideally, eDNA sampling should be carried out before trapping, as crayfish are drawn to the bait from their shelters and feeding activity combined with increased interactions may lead to higher rates of eDNA shedding. Nonetheless, we also observed higher turbidity in Lake Øymarksjøen than in Lake Stora Le. In Stora Le, non-detection of both *A. astaci* and *P. leniusculus* occurred only at locations with low CPUE (0.6 and 3.6) and the detected eDNA quantity corresponded well to the crayfish density. This also may be attributed to trapping prior to sampling as the data suggest from Øymarksjøen and other recent studies where no clear or only weak correlations were found between crayfish density and eDNA concentration (Dougherty et al. 2016; Cai et al. 2017; Larson et al. 2017; Rice et al. 2018; Johnsen et al. 2020). Generally, we detected eDNA of *A. astaci* at a higher frequency than that of *P. leniusculus*. One possible explanation for this may lie within the nature of the eDNA sampled. While eDNA from crayfish most likely consists of (dead) cell shedding in the water column, *A. astaci* eDNA is likely to be captured in the form of living zoospores and encysted spores which are less susceptible to immediate degradation caused by chemical and biological processes. Furthermore, compared to other aquatic organisms such as fish, crayfish seem to emit very low amounts of eDNA (Forsström and Vasemägi 2016; Fossøy et al. 2020; Johnsen et al. 2020).

Through the mesocosm experiment and the comparison with additional field data, we demonstrated that the detectability of both *P. leniusculus* and *A. astaci* eDNA is influenced by much more than mere population density. When sampling to monitor the presence of *A. astaci*, it is advisable to analyse the samples for eDNA of both the host and the pathogen for optimal detection efficiency. The crayfish plague agent *A. astaci* requires a crayfish host (or another freshwater decapod crustacean, see Schrimpf et al. 2014; Svoboda et al. 2014; Putra et al. 2018) for long term survival (OIE 2019). Moreover, only few studies report on NICS populations that are free of infections with *A. astaci* or below the level of detection (Schrimpf et al. 2013; Tilmans et al. 2014; Mojžišová et al. 2022). An efficient sampling strategy requires both a robust knowledge of the biology of the target species (Rusch et al. 2020) as well as taking spatio-temporal considerations into account (Thalinger et al. 2021). Furthermore, the number of samples heavily impacts the success of detection. Through our statistical modelling, we show that a high detection probability is dependent on crayfish density (CPUE). For *P. leniusculus*, the required density was 5 CPUE in Stora Le (based on ddPCR results, Fig. 7B) and 3 CPUE in Øymarksjøen (based on qPCR results, Fig. 7C) for a near 100% detection probability in only one sample. It is not uncommon to find crayfish populations with markedly lower population densities (Johnsen et al. 2020) where one sample would be insufficient for a positive detection. Other studies report similar results where crayfish are detected at low densities, but with only infrequent positive detection (Dougherty et al. 2016; Larson et al. 2017; Johnsen et al. 2020). For *A. astaci* in the two lakes studied by us, required crayfish density for a near 100% detection

probability was 2 CPUE. This number may be subject to variation, depending on the infection status, *A. astaci* prevalence and agent level (Vrålstad et al. 2009; Strand et al. 2014). Statistical modelling of the required sampling effort is, therefore, highly advisable (Dougherty et al. 2016; Johnsen et al. 2020; Sieber et al. 2020).

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Supplementary material 1

Primers and probes used in the present study

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (pdf file)

Explanation note: Primers and probes for *Aphanomyces astaci* and signal crayfish (*Pacifastacus leniusculus*) used in the present study.

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Link: https://doi.org/10.3897/neobiota.79.82793.suppl1

Supplementary material 2

ddPCR and qPCR data from the mesocosm experiments

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (excel file)

Explanation note: ddPCR and qPCR data from the mesocosm experiments at 20° C ("warm") (EXP1-EXP3). ddPCR and qPCR data from the mesocosm experiments at 10° C ("low temp") (EXP4-EXP6).

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Link: https://doi.org/10.3897/neobiota.79.82793.suppl2

Supplementary material 3

R-Script of GLM analysis
Authors: Johannes Rusch, David Strand, Tom Andersen
Data type: statistics
Explanation note: R-Script of GLM analysis of data from the mesocosm experiments.
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Supplementary material 4

R-script of MCOCC occupancy analysis
Authors: Johannes Rusch, David Strand, Tom Andersen
Data type: statistics
Explanation note: R-script of the MCOCC occupancy analysis for field data.
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Link: https://doi.org/10.3897/neobiota.79.82793.suppl4

Supplementary material 5

Results of the GLMM model
Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad
Data type: statistics
Explanation note: Results of the GLMM model for *A. astaci* (*A. ast*) and signal crayfish (*P. len*) determining statistical significance of three factors (food, temperature, density) on quantity of eDNA represented by positive droplets.
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Link: https://doi.org/10.3897/neobiota.79.82793.suppl5

Supplementary material 6

Agent levels of *Aphanomyces astaci* in individuals of *Pacifastacus leniusculus* used in the experiment

Authors: Johannes C. Rusch, David A. Strand, Charlotte Laurendz, Tom Andersen, Stein I. Johnsen, Lennart Edsman, Trude Vrålstad

Data type: table (pdf file)

Explanation note: Agent levels of *Aphanomyces astaci* in individuals of *Pacifastacus leniusculus* used in the experiment. The agent level categories (A0-A5; Vrålstad et al. 2009) are based on DNA copy numbers or PFU (PCR forming units) obtained from qPCR analysis of signal crayfish tissue samples.

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